THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 18, Issue of April 30, pp. 18840–18850, 2004
Printed in U.S.A.

Hetero-oligomerization between $GABA_A$ and $GABA_B$ Receptors Regulates $GABA_B$ Receptor Trafficking*

Received for publication, December 9, 2003, and in revised form, February 10, 2004
Published, JBC Papers in Press, February 13, 2004, DOI 10.1074/jbc.M313470200

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The neurotransmitter γ-aminobutyric acid (GABA) mediates inhibitory signaling in the brain via stimulation of both $GABA_A$ receptors ($GABA_AR$), which are chloride-permeant ion channels, and $GABA_B$ receptors ($GABA_BR$), which signal through coupling to G proteins. Here we report physical interactions between these two different classes of GABA receptor. Association of the $GABA_B$ receptor 1 ($GABA_BR1$) with the $GABA_A$ receptor γ2S subunit robustly promotes cell surface expression of $GABA_BR1$ in the absence of $GABA_BR2$, a closely related $GABA_B$ receptor that is usually required for efficient trafficking of $GABA_BR1$ to the cell surface. The $GABA_BR1$/γ2S complex is not detectably functional when expressed alone, as assessed in both ERK activation assays and physiological analyses in oocytes. However, the γ2S subunit associates not only with $GABA_BR1$ alone but also with the functional $GABA_BR1$/$GABA_BR2$ heterodimer to markedly enhance $GABA_B$ receptor internalization in response to agonist stimulation. These findings reveal that the $GABA_BR1$/γ2S interaction results in the regulation of multiple aspects of $GABA_B$ receptor trafficking, allowing for cross-talk between these two distinct classes of GABA receptor.

$GABA$,[1] the primary inhibitory neurotransmitter in the mammalian brain, produces its physiological effects by acting on three different receptor subtypes: $GABA_A$, $GABA_B$, and $GABA_C$ (1). The ionotropic receptors $GABA_A$ and $GABA_C$ produce fast inhibitory synaptic transmission via an intrinsic chloride channel. $GABA_A$ receptors are pentamers composed of combinations of various subunits, with the most prevalent combination in the mammalian brain containing two α1 subunits, two β2 subunits, and one γ2 subunit (2). $GABA_B$ receptors, in contrast, are metabotropic G protein-coupled receptors (GPCRs) that mediate the slow inhibitory neurotransmission of GABA via the regulation of several effectors. $GABA_B$ receptors are believed to be heterodimeric combinations of two GPCRs, $GABA_BR1$ and $GABA_BR2$ (3–5). $GABA_BR1$, the first receptor to be cloned, was found to bind GABA with low affinity and couple much less efficiently to effectors than native $GABA_B$ receptors (6). It was soon established that $GABA_BR1$, when expressed alone in heterologous systems, could not traffic efficiently to the cell surface but was rather retained in the endoplasmic reticulum (ER) due to the presence of an ER retention motif on its intracellular C terminus (7, 8). A second receptor, $GABA_BR2$, was subsequently cloned and found to be capable of trafficking to the cell surface by itself yet incapable of binding ligand or coupling to G proteins. When $GABA_BR1$ and $GABA_BR2$ were co-expressed in heterologous cells, they were found to form functional surface-expressed receptors with properties similar to those of some native $GABA_B$ receptors (9–11). In the heterodimer, $GABA_BR1$ is thought to bind the ligand (12), whereas $GABA_BR2$ is believed to be the primary G protein contact site (13–15).

Despite the recent advances in the understanding of $GABA_B$ receptors at the molecular level, several puzzling facts remain. First, despite the apparent functional requirement for heterodimerization, $GABA_BR1$ is distributed in many regions of the brain (for example, the anterior pituitary and interneurons of the hippocampus) and periphery (uterus, spleen) that show no $GABA_BR2$ expression but have demonstrable GABA binding and responses (16–18). Second, native $GABA_B$ receptors exhibit tremendous ligand binding heterogeneity, which splice variants of the cloned receptors $GABA_BR1$ and $GABA_BR2$ do not adequately explain (4, 19–22). Finally, numerous reports suggest that $GABA_B$ receptors participate in physiological cross-talk with other receptors through unknown mechanisms. Of particular note are functional interactions between $GABA_A$ and $GABA_B$ receptors in regulating each other's binding properties (23–25) and activity (24, 26–29).

In recent years, heterodimerization of receptors has helped to explain some examples of pharmacological heterogeneity and cross-talk between other neurotransmitter receptors (30, 31). For example, heterodimerization of κ and δ opioid receptors results in a new receptor with distinct pharmacological properties (32). Furthermore, physiologically important heterodimerization has been demonstrated not only between GPCRs, but also between GPCRs and ionotropic receptors. For example, $GABA_A$ receptors have been shown to physically interact with dopamine receptor 5 (33), whereas NMDA-type glutamate receptors have been found to associate with dopamine receptor 1 (34, 35), leading to mutual regulation of receptor function.

* This work was supported by National Institutes of Health Grant R01-NS45644, a Distinguished Young Scholar in Medical Research award from the W. M. Keck Foundation (to R. A. H.), and an NRSA post-doctoral award from the National Institutes of Health (to J. A. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GABA, γ-aminobutyric acid; $GABA_AR$, $GABA_A$ receptor; $GABA_BR1$, $GABA_B$ receptor 1; $GABA_BR2$, $GABA_B$ receptor 2; GPCR, G protein-coupled receptor; ER, endoplasmic reticulum; NMDA, *N*-methyl-D-aspartate; γ2S, γ2 short; γ2L, γ2 long; HA, hemagglutinin; HEK-293, human embryonic kidney 293 cells; mGluR, metabotropic glutamate receptor; LPA, lysophosphatidic acid receptor; H, histamine receptor; GST, glutathione *S*-transferase; CT, C terminus; ICL, intracellular loops; ERK, extracellular-signal-regulated kinase; GIRK, G protein-activated inwardly rectifying potassium channel; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

We hypothesized that $GABA_BR1$ might potentially associate with other receptors and that this might account in part for certain aspects of $GABA_B$ receptor function that are not presently understood. This idea led us to screen a library of different receptors as possible trafficking partners for $GABA_BR1$. Surprisingly, these screens revealed that co-expression with the γ2S subunit of the $GABA_A$ receptor produced robust cell surface expression of $GABA_BR1$ in the absence of $GABA_BR2$. Furthermore, we found that there is a physical interaction between discrete regions of $GABA_BR1$ and the $GABA_A$ receptor γ2S subunit, and that this association has significant functional consequences for $GABA_B$ receptor trafficking and endocytosis.

MATERIALS AND METHODS

Plasmids—Epitope-tagged (HA-, FLAG-, Myc-, and His-tagged) versions of human $GABA_BR1b$ and $GABA_BR2$ in the mammalian expression vector pcDNA3.1 were kindly provided by Fiona Marshall (GlaxoSmithKline). β_1- and β_2-adrenergic receptor constructs were kindly provided by Robert Lefkowitz (Duke University Medical Center). α_{1A}-, α_{1B}-, and α_{1D}-adrenergic receptor constructs were kindly provided by Ken Minneman (Emory University School of Medicine). α_{2A}-, α_{2B}-, and α_{2C}-adrenergic receptor constructs were kindly provided by Lee Limbird (Vanderbilt University Medical Center). Dopamine receptor 1 and 2 constructs were kindly provided by David Sibley (National Institutes of Health). The serotonin 5HT1A receptor construct was kindly provided by John Raymond (Medical University of South Carolina). Angiotensin AT1 and AT2 receptor constructs were kindly provided by Victor Dzau (Harvard Medical School). Muscarinic m1–5 acetylcholine receptor constructs were kindly provided by Allan Levey (Emory University School of Medicine). Opioid receptor constructs (μ, δ, and κ) were kindly provided by Lakshmi Devi (New York University School of Medicine) and Ping-Yee Law (University of Minnesota Medical School). Lysophosphatidic acid-1 and -2 receptor constructs were kindly provided by Jerold Chun (University of California, San Diego). Histamine H1–4 receptor constructs were kindly provided by Tim Lovenberg (The R. W. Johnson Pharmaceutical Research Institute). Metabotropic glutamate receptor constructs (mGluR1–8) were kindly provided by Jeff Conn (Emory University School of Medicine). NMDA receptors 1A and 2A constructs were kindly provided by Steve Traynelis (Emory University School of Medicine). $GABA_A$ receptor α1, β2, γ2S, and γ2L subunit constructs were kindly provided by Cynthia Czajkowski (University of Wisconsin at Madison) and Susan M. J. Dunn (University of Alberta). GIRK1 and GIRK4 constructs were kindly provided by David Mott (Emory University).

Cell Culture and Transfection—All tissue culture media and related reagents were purchased from Invitrogen. HEK-293 cells were maintained in complete medium (Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 1% penicillin/streptomycin) in a 37 °C, 5% CO_2 incubator. For heterologous expression of receptors, 2–4 μg of cDNA was mixed with LipofectAMINE (15 μl), and Plus reagent (10 μl) (Invitrogen) and added to 5 ml of serum-free medium in 10-cm tissue cultures plates containing cells at 60–80% confluency. Following a 4-h incubation, 6 ml of fresh complete medium was added. After another 12–16 h incubation, the medium was changed again, and the cells were harvested 24 h later.

Western Blotting—Samples (5 μg per lane) were run on 4–20% SDS-PAGE (Invitrogen) for 1 h at 180 V and then transferred to nitrocellulose. The blots were blocked in "blot buffer" (2% non-fat dry milk, 0.1% Tween 20, 50 mM NaCl, 10 mM HEPES, pH 7.4) for at least 30 min and then incubated with an appropriate primary antibody in blot buffer for 1 h at room temperature. The blots were then washed three times with 10 ml of blot buffer and incubated for 1 h at room temperature with an appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences and Chemicon) in blot buffer. Finally, the blots were washed three more times with 10 ml of blot buffer and visualized via enzyme-linked chemiluminescence using the ECL kit from Amersham Biosciences or Pierce.

Antibodies—The primary antibodies utilized were 12CA5 monoclonal anti-HA antibody (Roche Applied Science), M2 monoclonal anti-FLAG antibody (Sigma), monoclonal anti-c-Myc 9E10 antibody (Sigma), anti-$GABA_BR1$ antibody, anti-$GABA_BR2$ antibody (Chemicon), anti-$GABA_Aα1$ antibody, anti-$GABA_Aβ2$ antibody (Upstate Biotechnology), and anti-$GABA_Aγ2$ antibody (Alpha Diagnostic International).

Surface Expression Assay—HA-$GABA_BR1$ cDNA was transfected either alone or with other receptor cDNAs. Transfected HEK-293 cells were plated in 35-mm tissue culture plates at 80% confluency. 24 h later, the cells were fixed in 4% paraformaldehyde in PBS/Ca^{2+} for 30 min and then blocked with "cell blocking buffer" (2% non-fat dry milk in PBS/Ca^{2+}) for 30 min at room temperature. They were then incubated with a monoclonal 12CA5 anti-HA antibody for 1 h to detect the HA-$GABA_BR1$ on the cell surface. Following three washes (5 min) with cell blocking buffer, the cells were incubated with horseradish peroxidase-coupled anti-mouse secondary antibody (Amersham Biosciences) for 1 h. The cells were washed three times for 5 min with cell blocking buffer, twice with PBS, and then developed with 2 ml of enzyme-linked immunosorbent assay SuperSignal Pico ECL from Pierce for exactly 15 s. Chemiluminescence of the whole 35-mm plate was quantified in a TD20/20 luminometer (Turner Designs). For each data point, 3–5 dishes were averaged per experiment. The results were analyzed using one-way analysis of variance and Dunnett's post hoc tests where applicable (GraphPad Prism). For each transfection condition in each surface expression experiment, matching dishes of transfected cells were harvested and examined via Western blot to confirm the expression of the various receptors involved.

Double Immunofluorescence Microscopy—HEK-293 cells were transiently transfected with HA-$GABA_BR1$ alone, HA-$GABA_BR1$/FLAG-$GABA_BR2$, or HA-$GABA_BR1$/Myc-$GABA_Aγ2S$. Transfected cells were plated in slides, fixed with 4% paraformaldehyde, and permeabilized with "saponin buffer" containing 2% bovine serum albumin and 0.04% saponin in PBS for 30 min at room temperature. The cells were then incubated with M2 monoclonal anti-FLAG antibody (Sigma), monoclonal anti-c-Myc 9E10 antibody (Sigma), and anti-$GABA_BR1$ antibody (Chemicon) for 1 h at room temperature. After three washes (1 min) with saponin buffer, the cells were incubated with a rhodamine red-conjugated anti-mouse IgG at 1:200 dilution and FITC-conjugated anti-guinea pig IgG at 1:200 dilution (Jackson ImmunoResearch) for 1 h at room temperature. After three washes (1 min) with saponin buffer and one wash with PBS, coverslips were mounted, and rhodamine red-labeled FLAG-$GABA_BR2$ or Myc-$GABA_Aγ2S$ and FITC-labeled HA-$GABA_BR1$ were visualized with a Zeiss LSM-510 laser confocal microscope. Multiple control experiments, utilizing either transfected cells in the absence of primary antibody or untransfected cells in the presence of primary antibody, revealed a very low level of background staining, indicating that the primary antibody-dependent immunostaining observed in the transfected cells was specific.

Immunoprecipitation—Cells were harvested and lysed in 500 μl of ice-cold lysis buffer (10 mM HEPES, 50 mM NaCl, 1.0% Triton X-100, 5 mM EDTA, and the protease inhibitor mixture from Roche Applied Science). The lysate was solubilized via end-over-end rotation at 4 °C for 30 min and clarified via centrifugation at 14,000 rpm for 15 min. A small fraction of the supernatant was taken at this point and incubated with SDS-PAGE sample buffer in order to examine expression of proteins in the whole cell extract. The remaining supernatant was incubated with 4–8 μg of respective antibody and 50 μl of protein A/G-agarose beads (Oncogene) or 50 μl of beads covalently linked to anti-FLAG antibodies (Sigma) or anti-HA antibodies (Covance) for 2 h with end-over-end rotation at 4 °C. After five washes with 1 ml of lysis buffer, the immunoprecipitated proteins were eluted from the beads with 1× SDS-PAGE sample buffer, resolved by SDS-PAGE, and subjected to Western blot analyses.

GST Pull-down Assay—$GABA_BR1$ C terminus and truncations were prepared with a hexahistidine C-terminal tag via PCR amplification of full-length rat $GABA_BR1$ and subcloned into the pGEX-4T1 vector (Amersham Biosciences) using EcoRI and XhoI restriction enzymes. GST α1 ICL and GST β2 ICL were generous gifts from Cynthia Czajkowski (University of Wisconsin, Madison). GST γ2S ICL containing 86 amino acids was PCR-amplified from full-length γ2S construct and subcloned into pGEX-4T1 vector using EcoRI and XhoI restriction enzymes. Fusion proteins grown in *E. coli* were isolated and purified on glutathione-agarose beads. Aliquots of the fusion protein on beads were blocked 30 min with 1 ml of "BSA buffer" (3% BSA, 10 mM HEPES, 50 mM NaCl, 0.1% Tween 20) at 4 °C. Solubilized brain lysates or lysates from transfected HEK-293 cells were then incubated with the beads end-over-end at 4 °C overnight. Following three washes with 1 ml of BSA buffer, the proteins were eluted off the beads with sample buffer, resolved on SDS-PAGE gels, and analyzed via Western blot using appropriate antibodies.

ERK Activation Assay—HEK-293 cells were transfected with HA-$GABA_BR1$ alone, HA-$GABA_BR1$/FLAG-$GABA_BR2$, HA-$GABA_BR1$/Myc-$GABA_Aγ2S$, or HA-$GABA_BR1$/$GABA_Aα1β2γ2S$. Transfected HEK-293 cells were plated in 35-mm tissue culture plates at 80% confluency and serum-starved overnight the day before the assay. The cells were stimulated with 100 μM baclofen, rinsed with ice-cold PBS/Ca^{2+}, and lysed

in 150 μl of sample buffer. The cell lysates were run on SDS-PAGE gels and then analyzed via Western blotting with anti-phospho-p44/42 MAPK and anti-p44/42 MAPK antibodies (Cell Signaling).

Electrophysiology—Stage V–VI oocytes were harvested from *Xenopus laevis* and prepared for injection. Single oocytes were injected within 24 h with 5 nl of cRNA prepared from cDNA using RNA Express kit (Ambion) (1 ng to 1 μg/μl per subunit) and were assayed functionally at least 2 days after cRNA injection. GABA$_A$ receptor-mediated currents were measured from oocytes perfused with "ND96/Ca^{2+}" (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.2) in a recording chamber. Recordings were made using 2-electrode voltage clamp while holding the oocytes at −80 mV with borosilicate electrodes filled with 300 mM KCl. GABA$_A$ receptor currents were measured by perfusing GABA (Tocris) or GABA plus diazepam (Sigma) directly onto oocytes under clamp conditions. GIRK current measurement was made in an identical set up, but the recordings were made in "40K solution" (60 mM NaCl, 40 mM KCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 6 mM HEPES, pH 7.4), and oocytes were clamped at a voltage of −100 mV. GABA (Tocris) or baclofen (Sigma) was dissolved in 40K solution and applied by bath superfusion.

Electrophysiology Analysis—The current peaks were measured using mini-analysis (Synaptosoft) and analyzed with GraphPad Prism. All concentration-response data were fit by Equation 1,

$$I = I_{max} \times [L]^n / [L]^n \times [EC_{50}]^n \quad \text{(Eq. 1)}$$

where I is the current response; I_{max} is the maximal current response; [L] is the drug concentration; EC_{50} is the drug concentration that evokes half-maximal current response; and n is the Hill coefficient. The diazepam potentiation of I_{GABA} was defined as shown in Equation 2,

$$P = [I_{GABA\ +\ diazepam} / I_{GABA}] - 1 \quad \text{(Eq. 2)}$$

where $I_{GABA+diazepam}$ is the current response in the presence of GABA and diazepam, and I_{GABA} is the current evoked solely by GABA. Diazepam potentiation was measured at a low concentration (10 μM) of GABA (EC_2–EC_{10}). The results were statistically compared using unpaired t tests as required.

Internalization Assay—HEK-293 cells were transfected with HA-GABA$_B$R1/FLAG-GABA$_B$R2 or HA-GABA$_B$R1/FLAG-GABA$_B$R2 + Myc-GABA$_A$γ2S. Transfected cells were plated in 35-mm tissue culture plates at 80% confluency. On the day of the assay, the cells were stabilized at room temperature for 2 h and stimulated with 100 μM GABA for 30 min at room temperature, and the amount of GABA$_B$R1 on the cell surface was assayed in a manner identical to the surface expression assay described above.

RESULTS

The GABA$_A$ Receptor γ2S Subunit Promotes GABA$_B$R1 Cell Surface Expression—To screen for potential interacting partners that might aid in trafficking GABA$_B$R1 to the plasma membrane, we used a cell surface expression assay that has been used previously to study GABA$_B$R1 plasma membrane expression (7). HA-tagged GABA$_B$R1 was sequentially co-expressed with various GPCRs and ionotropic receptors in HEK-293 cells, and the cell surface expression of GABA$_B$R1 was analyzed using a luminometer-based assay. In this assay, GABA$_B$R1 expressed alone showed barely detectable cell surface expression, whereas co-expression with GABA$_B$R2 yielded a nearly 40-fold increase in GABA$_B$R1 cell surface expression, as reported previously (3, 7). GABA$_B$R1 was also co-expressed in these screens with 34 other GPCRs. In contrast to GABA$_B$R2, none of these receptors significantly facilitated GABA$_B$R1 surface expression. It has been reported previously that co-expression with the metabotropic glutamate receptor mGluR4 may (36) or may not (8) have a modest effect on GABA$_B$R1 surface expression, but our studies consistently revealed no effect of any of the mGluR subtypes on trafficking of GABA$_B$R1 to the cell surface. Similarly, co-expression with the NMDA-type glutamate receptor subunits NR1 and -2A also failed to enhance the surface expression of GABA$_B$R1. Strikingly, however, co-expression with either the γ2S subunit of the GABA$_A$ receptor or the entire GABA$_A$ receptor complex composed of α1, β2, and γ2S subunits increased the surface expression of GABA$_B$R1 by ~15–20-fold (Fig. 1). Because co-expression with the α1 and β2 subunits alone had no effect on GABA$_B$R1 surface expression, these results revealed that the γ2S subunit is capable of trafficking GABA$_B$R1 to the cell surface in the absence of GABA$_B$R2.

The surprising effect of γ2S subunit co-expression on GABA$_B$R1 plasma membrane trafficking was confirmed via immunocytochemistry. HEK-293 cells were transfected with either (i) GABA$_B$R1 alone, (ii) differentially tagged GABA$_B$R1 and GABA$_B$R2, or (iii) differentially tagged GABA$_B$R1 and GABA$_A$ receptor γ2S subunit. The receptors were labeled with specific fluorescent antibodies and visualized via confocal microscopy. These co-localization studies revealed that GABA$_B$R1 was diffusely distributed throughout the cytoplasm when expressed alone (Fig. 2, *A–C*) but found predominantly at the plasma membrane when co-expressed with GABA$_B$R2 (Fig. 2, *D–F*). Remarkably, a similar localization of GABA$_B$R1 to the plasma membrane was observed following co-expression with the γ2S subunit (Fig. 2, *G–I*). These data confirm that the GABA$_A$ receptor γ2S subunit can mimic GABA$_B$R2 in its ability to promote the trafficking of GABA$_B$R1 to the cell surface and furthermore show that the γ2S subunit and GABA$_B$R1 are co-localized in the plasma membrane when co-expressed in HEK-293 cells.

GABA$_B$ Receptors and GABA$_A$ Receptor Subunits Physically Associate in Transfected Cells and Native Brain Tissue—The profound enhancement of GABA$_B$R1 surface expression induced by co-expression with the GABA$_A$ receptor γ2S subunit suggested that there might be a physical interaction between these two proteins. This possibility was examined via co-immunoprecipitation experiments. As shown in Fig. 3*A*, GABA$_B$R1 was robustly co-immunoprecipitated with the γ2S subunit from transfected HEK-293 cell lysates, revealing that the two proteins can indeed associate in a cellular context. The potential interactions of other GABA$_A$ receptor subunits with GABA$_B$R1 were also examined. It was found that GABA$_B$R1 could be co-immunoprecipitated from transfected HEK-293 cell lysates with α1 subunits (Fig. 3*B*) but not with β2 subunits (Fig. 3*C*). Moreover, GABA$_B$R1 was still found to associate with γ2S and α1 subunits even in the presence of GABA$_B$R2. Similarly, GABA$_B$R2 could be co-immunoprecipitated with γ2S and α1 subunits but not with β2 subunits (Fig. 3, *A–C, bottom panels*) both in the presence and absence of GABA$_B$R1. We also conducted reverse experiments to see whether GABA$_A$ receptors were co-immunoprecipitated with GABA$_B$R1. As shown in Fig. 3*D*, immunoprecipitation of FLAG-GABA$_B$R1 from cells transfected with α1β2γ2S resulted in robust co-immunoprecipitation of all of the GABA$_A$ receptor subunits. The extent of α1 subunit co-immunoprecipitation with GABA$_B$R1 was similar for α1 alone (*10th lane*) *versus* α1β2 (*9th lane*) *versus* α1β2γ2S (*8th lane*). These data suggest that both GABA$_B$R1 and GABA$_B$R2 can associate with functional GABA$_A$ receptor pentamers as well as with the individual subunits α1 and γ2S. Finally, we examined the potential association of endogenous GABA$_B$ receptors and GABA$_A$ receptor γ2 subunits in native brain tissue. As shown in Fig. 3*E*, GABA$_B$ receptors were strongly co-immunoprecipitated with γ2 subunits from solubilized rat brain lysates, demonstrating that GABA$_B$ receptors and GABA$_A$ receptor γ2 subunits form complexes not only in transfected cells but also in native brain tissue.

GABA$_B$R1 Surface Expression Is Promoted by γ2S but Not α1β2 or γ2L Subunits—The co-immunoprecipitation experiments revealed that both α1 and γ2S subunits can associate with GABA$_B$R1. These findings were somewhat surprising, because our initial screens had indicated that the α1 subunit was unable to promote GABA$_B$R1 trafficking to the plasma

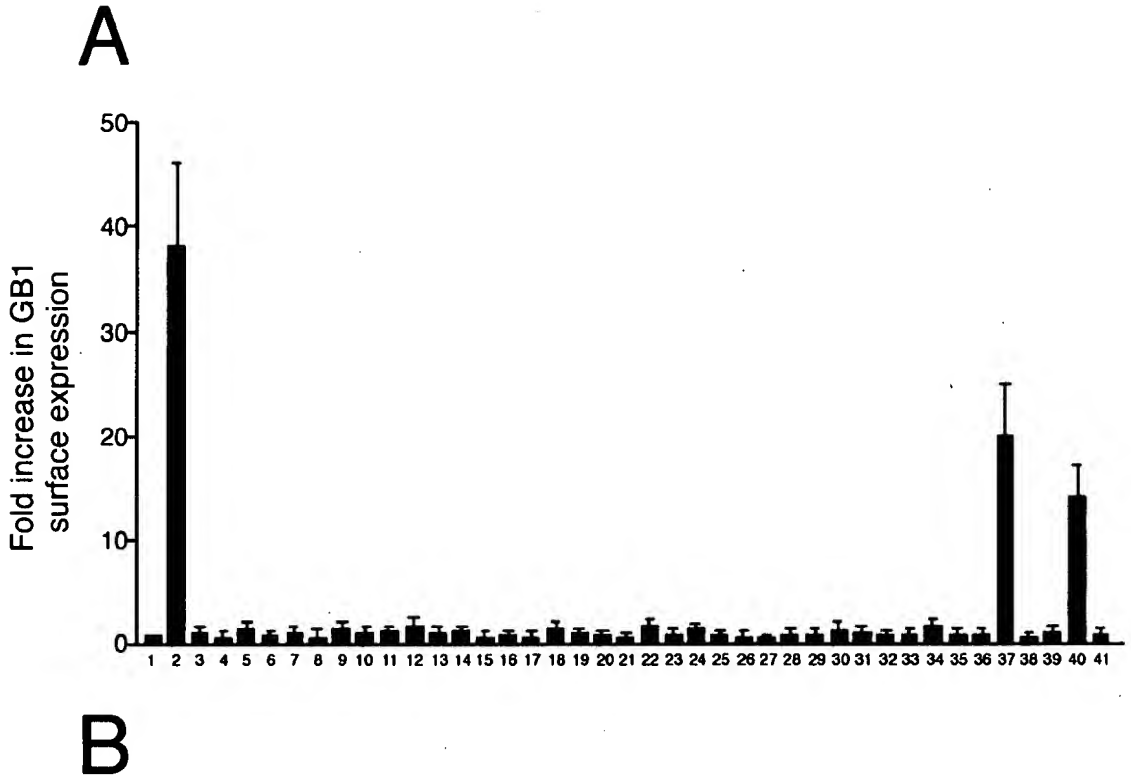

FIG. 1. **$GABA_BR1$ is trafficked to the cell surface by $GABA_A\gamma2S$ but not by other receptors.** HA-$GABA_BR1$ surface expression was detected and quantified by a luminometer-based assay (*A*) following HA-$GABA_BR1$ co-expression in HEK-293 cells with other GPCRs and ligand-gated ion channels listed as *1–41* (*B*). The receptors examined in this study were chosen because they are known to be expressed in at least some of the same brain regions as $GABA_BR1$. In these experiments, $GABA_BR1$ expressed by itself was barely detectable on the cell surface, whereas $GABA_BR1$ co-expressed with $GABA_BR2$ as a positive control showed a nearly 40-fold increase in surface expression over $GABA_BR1$ alone. Co-expression with most of the other receptors examined had no significant effect on $GABA_BR1$ surface expression, but co-expression of $GABA_BR1$ with the $GABA_A$ receptor γ2S subunit or with the whole $GABA_A$ receptor complex containing the γ2S subunit showed a 15–20-fold increase in surface expression. The *bars* and *error bars* represent mean ± S.E. for 3–5 independent experiments. Abbreviations are as follows: *GB*, $GABA_B$ receptor; *AR*, adrenergic receptor; *DR*, dopamine receptor; *5HTR*, serotonin receptor; *ATR*, angiotensin receptor; *mAChR*, muscarinic acetylcholine receptor; *OR*, opioid receptor; *LPA*, lysophosphatidic acid receptor; *H*, histamine receptor; *mGluR*, metabotropic glutamate receptor.

membrane. One potential explanation for this result could be that the α1 subunit cannot traffic $GABA_BR1$ to the cell surface because it cannot access the cell surface by itself. It has been shown that the α1 and β2 subunits expressed individually in HEK-293 cells are retained in the endoplasmic reticulum, whereas α1 and β2 subunits expressed together form functional channels on the cell surface (37). Hence, we investigated the ability of α1β2 receptors to traffic $GABA_BR1$ to the cell surface. We found, however, that $GABA_BR1$ exhibited no significant increase in trafficking to the plasma membrane when co-expressed with α1β2 receptors (Fig. 4*A*). In contrast, co-expression of $GABA_BR1$ with the α1β2γ2S $GABA_A$ receptor resulted in a more than 20-fold enhancement in $GABA_BR1$ surface expression, as shown earlier. In matching control experiments, we found that $GABA_A$ receptor α1 subunits were efficiently trafficked to the cell surface when expressed with β2 subunits but not when expressed alone (data not shown), consistent with previous observations (37). These data reveal that α1 and β2 subunits do not promote $GABA_BR1$ cell surface expression and that the γ2S subunit is required for this effect.

There are two known splice variants of the γ2 subunit, γ2S and γ2L, which differ in that γ2L has an additional 8 amino acids on one of its intracellular loops. We examined the ability of these two splice variants to regulate $GABA_BR1$ subcellular localization. As shown earlier, co-expression of $GABA_BR1$ with the γ2S subunit resulted in a more than 15-fold increase in $GABA_BR1$ surface expression. In contrast, co-expression of $GABA_BR1$ with the γ2L subunit had absolutely no effect at all on the amount of $GABA_BR1$ trafficked to the cell surface (Fig. 4*B*). This may be attributed to the fact that the γ2S subunit can traffic to the cell surface when expressed alone in heterologous cells, although it does not form functional receptors (38). The γ2L subunit, conversely, is retained in the endoplasmic reticulum when expressed alone and is trafficked to the cell surface only when expressed with α and β subunits to form fully functional $GABA_A$ receptors (38). These findings demonstrate that

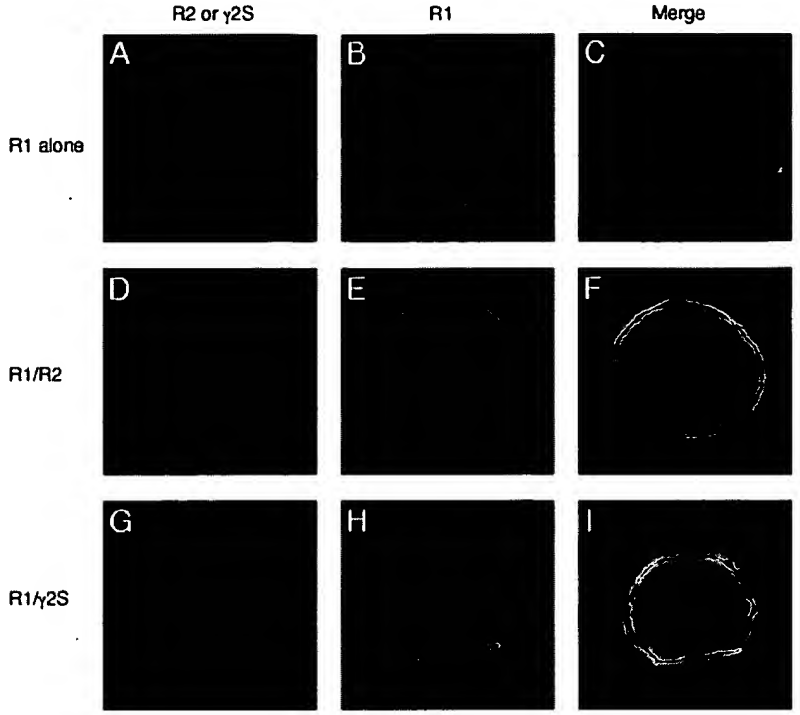

FIG. 2. **Co-expression of GABA$_B$R1 with the GABA$_A$ receptor γ2S subunit results in plasma membrane expression and co-localization of GABA$_B$R1/γ2S.** HA-GABA$_B$R1 expressed alone in HEK cells and visualized with a FITC-conjugated antibody exhibited diffuse intracellular staining (*A–C*). When HA-GABA$_B$R1 (FITC) was expressed with FLAG-GABA$_B$R2, which was visualized with rhodamine red, it was concentrated at the plasma membrane along with GABA$_B$R2 (*D–F*). Similarly, co-expression of HA-GABA$_B$R1 (FITC) with Myc-GABA$_A$γ2S (rhodamine red) also resulted in striking co-localization and membrane targeting of both receptors (*G–I*). The data presented here are representative of three independent experiments.

the two GABA$_A$ receptor γ2 subunit splice variants exert radically different effects on GABA$_B$R1 trafficking, with only γ2S being competent to promote GABA$_B$R1 cell surface expression.

The C terminus of GABA$_B$R1 Is Sufficient but Not Necessary for Association with GABA$_A$ Receptors—In order to elucidate the structural determinants of the interaction between GABA$_B$R1 and GABA$_A$ receptor subunits, we constructed a glutathione *S*-transferase (GST) fusion protein corresponding to the GABA$_B$R1 C terminus (CT), which is the largest cytoplasmic domain of the receptor (amino acids 856–960 of the full-length polypeptide). We then assessed the ability of this fusion protein to interact physically with full-length GABA$_A$ receptor subunits. The GABA$_B$R1-CT was able to pull down GABA$_A$ receptor subunits from solubilized rat brain lysates as detected by Western blotting with an antibody against the γ2 subunit (Fig. 5*A*, *1st 3 lanes*). Conversely, GST alone did not detectably pull down any GABA$_A$ receptor immunoreactivity. These data indicate that the C terminus of GABA$_B$R1 is sufficient to mediate an interaction with GABA$_A$ receptors. In order to pinpoint the exact region involved, GST fusion proteins representing three truncations of the C terminus of GABA$_B$R1 from the N-terminal end, corresponding to amino acids 879–960, 907–960, and 934–960, were prepared, and the ability of these truncants to pull down the GABA$_A$ receptor from solubilized brain lysates was examined. In these experiments, the two longest GABA$_B$R1-CT fusion proteins were capable of pulling down the GABA$_A$ receptor γ2 subunit, whereas the shortest fusion protein was not (Fig. 5*A*, *last 3 lanes*). These data indicated that the amino acids mediating the association are located between residues 907 and 934 of GABA$_B$R1. GST fusion proteins of five incremental truncations made between the region encompassed by amino acids 907–960 were then analyzed in a similar pull-down assay. In this experiment, addition of seven amino acids to the N terminus of the shortest fusion protein (934–960) of GABA$_B$R1 conferred the ability to pull down the GABA$_A$ γ2 subunit. Thus, these studies defined a region of seven amino acids in the GABA$_B$R1 C terminus (PPTPPDP) as a key determinant of the association with the GABA$_A$ receptor γ2 subunit (Fig. 5*B*).

In order to assess the requirement of the PPTPPDP motif for mediating the interaction between GABA$_B$R1 and the γ2S subunit, we prepared a mutant version of GABA$_B$R1 with the N terminus and the seven-transmembrane region intact but the C terminus truncated to remove the PPTPPDP motif and most of the GABA$_B$R1-CT. This truncated receptor was found to co-immunoprecipitate the GABA$_A$ receptor almost as well as wild-type GABA$_B$R1 (Fig. 5*C*). These findings suggest that the GABA$_B$R1 C terminus is sufficient but not necessary for interaction with the γ2S subunit and that other regions of GABA$_B$R1, such as perhaps the transmembrane domains, must be involved in the interaction. Similar results have been found for the interaction of GABA$_B$R1 with GABA$_B$R2, where the C termini of the receptors are clearly involved in the interaction but are by no means required (7, 8, 39).

Because the pull-down studies revealed that the intracellular C terminus of GABA$_B$R1 was sufficient for interaction with the γ2S subunit, we examined the intracellular regions of the GABA$_A$ receptor subunits for their ability to interact with GABA$_B$R1. GST fusion proteins corresponding to the intracellular loop (ICL) region between transmembrane 3 and transmembrane 4 of the α1, β2, and γ2S subunits were prepared and analyzed in a pull-down assay performed with lysates from cells transfected with GABA$_B$R1. As shown in Fig. 5*D*, the γ2S subunit ICL was capable of pulling down GABA$_B$R1, whereas GST alone, the α1 subunit ICL, and the β2 subunit ICL did not detectably pull down any GABA$_B$R1 immunoreactivity. These data demonstrate that the intracellular loop of the γ2S subunit is sufficient to mediate interaction with GABA$_B$R1.

Functional Consequences of Hetero-oligomerization between GABA$_A$ and GABA$_B$ Receptors—We hypothesized that the interaction between GABA$_B$R1 and the GABA$_A$ receptor γ2S subunit might serve as a point of cross-talk between GABA$_A$ and GABA$_B$ receptors and facilitate the mutual regulation of the receptors. To test this idea, we started by examining the responsivity of the GABA$_A$ receptor channel in *Xenopus* oocytes to GABA and benzodiazepines. Benzodiazepines are allosteric modulators of the GABA$_A$ receptor that increase the frequency of channel opening in the presence of GABA by binding to a

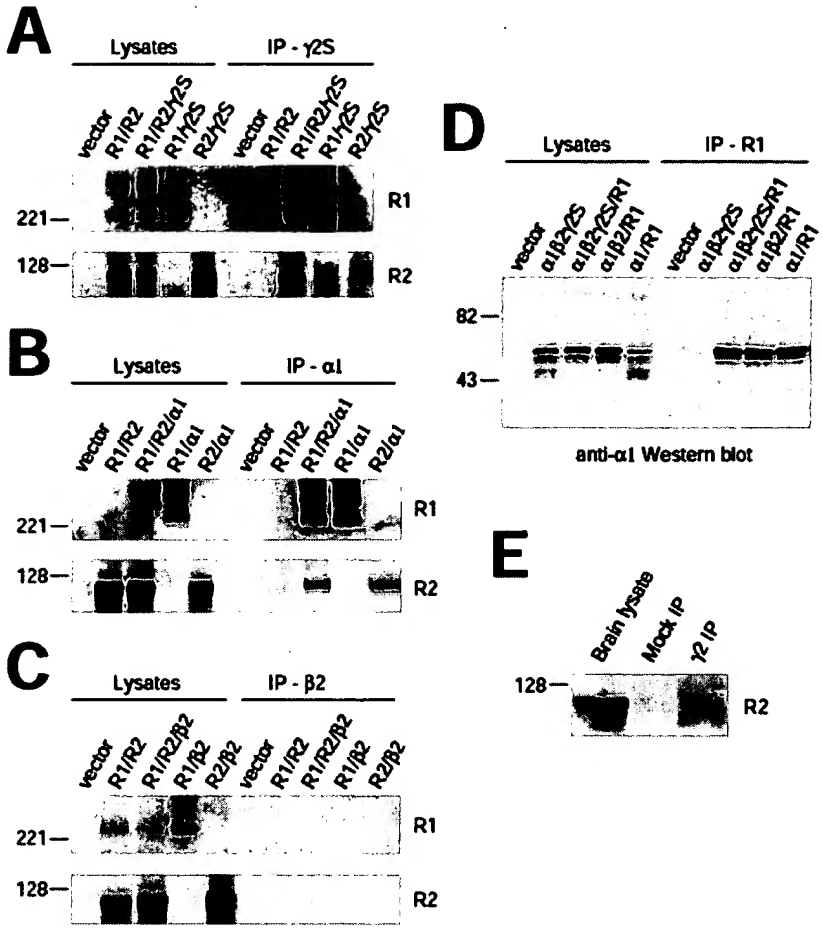

FIG. 3. **Co-immunoprecipitation of GABA$_A$ and GABA$_B$ receptors from transfected cells and native brain tissue.** *A*, HEK-293 cells were transfected with the following combinations of receptors: empty vector, GABA$_B$R1/GABA$_B$R2, GABA$_B$R1/GABA$_B$R2 plus Myc-GABA$_A$γ2S, GABA$_B$R1 plus Myc-GABA$_A$γ2S, and GABA$_B$R2 plus Myc-GABA$_A$γ2S. Cells were harvested, solubilized, and immunoprecipitated (*IP*) using an anti-Myc antibody. Western blot analysis revealed robust co-immunoprecipitation of GABA$_B$R1 and GABA$_B$R2 with the GABA$_A$ receptor γ2S subunit (*last 3 lanes*). GABA$_B$R1 immunoreactivity was usually observed as a higher molecular weight oligomer, as reported previously (9). *B*, HEK-293 cells were transfected with the same combinations of receptors as shown in *A*, except that Myc-GABA$_A$γ2S was replaced with FLAG-GABA$_A$α1. Western blot analysis following immunoprecipitation with anti-FLAG antibodies revealed that GABA$_B$R1 and GABA$_B$R2 co-immunoprecipitated with the GABA$_A$α1 subunit (*last 3 lanes*). *C*, HEK-293 cells were transfected with the same combinations of receptors as shown in *A* except that Myc-GABA$_A$γ2S was replaced with GABA$_A$β2 and immunoprecipitated with β2/3 antibody. However, neither GABA$_B$R1 nor GABA$_B$R2 was found to co-immunoprecipitate with the GABA$_A$β2 subunit (*last 3 lanes*). *D*, GABA$_B$R1 associates equally well with GABA$_A$ receptor subunits expressed individually or together. HEK-293 cells were transfected with either empty vector, α1β2γ2S, FLAG-GABA$_B$R1 plus GABA$_A$α1β2γ2S, FLAG-GABA$_B$R1 plus GABA$_A$α1β2 only, or FLAG-GABA$_B$R1 plus GABA$_A$α1 only. GABA$_B$R1 was immunoprecipitated with anti-FLAG antibody, and the co-immunoprecipitation of the GABA$_A$ receptor subunits was assessed. All three subunits were robustly co-immunoprecipitated (α1 is shown; β2 and γ2S are not shown), and the extent of α1 subunit co-immunoprecipitation with GABA$_B$R1 was consistently unchanged by the presence or absence of the other GABA$_A$ receptor subunits. *E*, GABA$_B$ receptors associate with GABA$_A$ receptor γ2 subunits in native brain tissue. Solubilized rat brain lysates were subjected to immunoprecipitation with either γ2 antibody (*γ2 IP*) or no antibody (*Mock IP*). Both GABA$_B$R1 (not shown) and GABA$_B$R2 were found to be specifically immunoprecipitated by the γ2 antibody. The data presented in all of the panels of this figure are representative of three to five independent experiments each.

unique site on the receptor protein (2). It is well established that the α and γ subunits confer benzodiazepine binding properties to GABA$_A$ receptors by forming a binding pocket at their interface (40). As both γ and α subunits were found in our studies to be involved in the association between GABA$_A$ and GABA$_B$ receptors, we monitored GABA$_A$ receptor currents and benzodiazepine responsiveness in the presence and absence of GABA$_B$R1 co-expression. Concentration response curves were constructed by measuring currents evoked by a low, non-saturating dose of GABA (10 μM) in the presence of varying concentrations of diazepam, a classical benzodiazepine. No significant differences in EC_{50} values for diazepam were found between GABA$_A$ receptors alone (α1β2γ2S; EC_{50} = 30.4 ± 1.7 nM, n = 5) and GABA$_A$ receptors co-expressed with GABA$_B$R1 (EC_{50} = 27.9 ± 6.2 nM, n = 9) (Fig. 6*A*). Thus, we found no evidence that co-expression with GABA$_B$R1 alters the sensitivity of GABA$_A$ receptors to diazepam modulation. We also examined whether co-expression with GABA$_B$R1 might affect the activation of GABA$_A$ channels by GABA. In these studies, a modest increase in GABA potency was observed for GABA$_A$ receptors co-expressed with GABA$_B$R1 (EC_{50} = 11.1 ± 1.7 μM, n = 10) compared with GABA$_A$ receptors expressed alone (EC_{50} = 18.7 ± 1.5 μM, n = 8) (Fig. 6*B*). These data suggest that agonist activation of GABA$_A$ receptors can be subtly modulated by the association of the receptors with GABA$_B$ receptors.

In addition to studying the effects of GABA$_B$ receptor co-expression on GABA$_A$ receptor function, we also examined the consequences of GABA$_A$ receptor co-expression on the function of GABA$_B$ receptors. It has been reported that GABA$_B$R1 is expressed at high levels in certain regions that lack detectable GABA$_B$R2 but have measurable responses to GABA (18). Thus, we explored the possibility that the GABA$_B$R1/γ2S complex might be functional in the absence of GABA$_B$R2. Two different types of experiments were conducted to address this question.

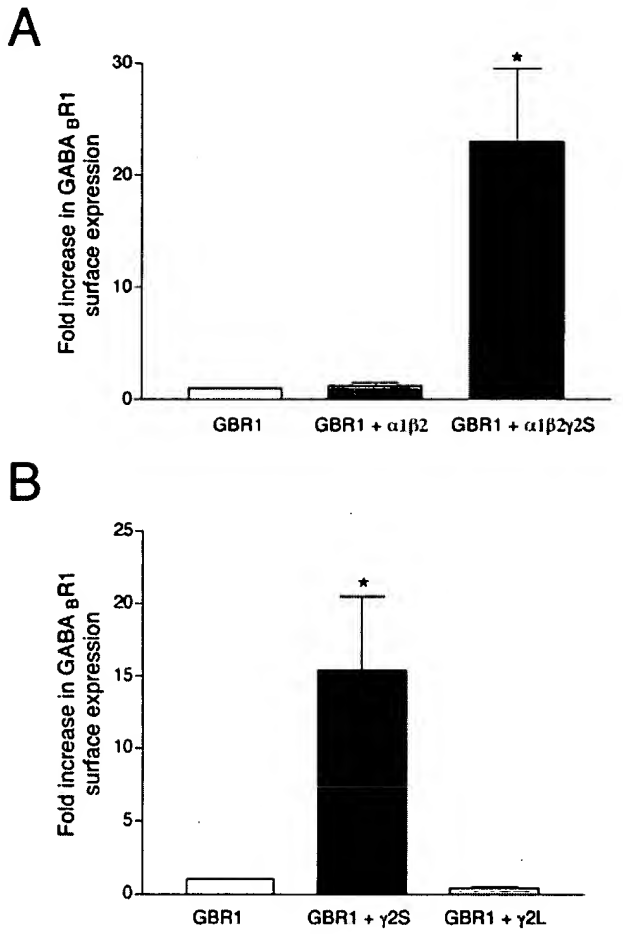

FIG. 4. **The γ2S subunit, but not other GABA$_A$ receptor subunits, can traffic GABA$_B$R1 to the cell surface.** *A*, GABA$_B$R1 surface expression in HEK-293 cells was assessed in the absence and presence of co-transfection with GABA$_A$ receptor α1β2 or α1β2γ2S subunits. Co-expression with α1β2 was found to be incapable of trafficking GABA$_B$R1 to the cell surface, whereas co-expression with α1β2γ2S strongly promoted the cell surface expression of GABA$_B$R1 (* = $p < 0.05$). *B*, the two γ2 subunit splice variants differ in their ability to traffic GABA$_B$R1 to the cell surface. GABA$_B$R1 surface expression was assessed in HEK-293 cells in the absence and presence of either γ2S or γ2L subunits. The γ2S splice variant promoted GABA$_B$R1 surface expression by more than 15-fold (* = $p < 0.05$), whereas the γ2L splice variant had no significant effect on GABA$_B$R1 surface expression. Expression levels of the two splice variants in these experiments were comparable, as assessed by Western blots (not shown). The data shown in both panels of this figure are representative of three independent experiments.

First, the ability of GABA$_B$ receptors to stimulate extracellular signal-regulated kinase (ERK) activity was measured, because many $G_{i/o}$-coupled receptors have been shown to activate the ERK pathway via G protein βγ subunits (41). ERK activation has not been shown yet for GABA$_B$ receptors, however; so we first performed time course studies to look for potential increases in ERK phosphorylation following GABA$_B$ receptor stimulation. HEK-293 cells were transfected with GABA$_B$ receptors (GABA$_B$R1 and GABA$_B$R2) and stimulated with 100 μM baclofen, a specific GABA$_B$ receptor agonist, for 1, 2, 5, 10, and 15 min. ERK activation was monitored by probing the cell lysates for phospho-ERK immunoreactivity on Western blots. To control for loading differences, Western blots for total ERK protein were also performed. As shown in Fig. 7*A*, GABA$_B$ receptor stimulation produced a profound increase in ERK phosphorylation at time points between 2 and 5 min. These data clearly demonstrate that the GABA$_B$ receptors are able to activate ERK following expression in HEK-293 cells. We next assessed if GABA$_B$R1 co-expressed with GABA$_A$ receptors was capable of activating ERK in the absence of GABA$_B$R2. HEK-293 cells were transfected with the following cDNA combinations: empty vector, GABA$_B$R1 alone, GABA$_B$R1/GABA$_B$R2, GABA$_B$R1/γ2S, or GABA$_B$R1/α1β2γ2S. The various sets of transfected cells were then stimulated with 100 μM baclofen for 5 min, and ERK activation was assessed via Western blots. Stimulation of cells transfected with empty vector or GABA$_B$R1 alone did not activate ERK, whereas stimulation of GABA$_B$R1/GABA$_B$R2 resulted in robust activation of ERK, as shown above. However, stimulation of cells transfected with GABA$_B$R1/γ2S or GABA$_B$R1/α1β2γ2S did not result in any detectable activation of ERK (Fig. 7*B*). These data suggest that although GABA$_B$R1 can be trafficked to the cell surface by co-expression with the GABA$_A$ receptor γ2S subunit, GABA$_B$R1 cannot stimulate ERK phosphorylation in the absence of GABA$_B$R2.

In a related set of experiments, we employed oocyte electrophysiology to test the physiological responsiveness of GABA$_B$R1/γ2S and GABA$_B$R1/α1β2γ2S complexes. It is well established that GABA$_B$ receptors can activate G protein-activated inwardly rectifying potassium (GIRK) channels in oocytes, whereas GABA$_B$R1 expressed in the absence of GABA$_B$R2 cannot independently activate GIRK channels (10). Oocytes were injected with cRNAs encoding GIRK1 and GIRK4 channels along with the following receptor combinations: GABA$_B$R1 alone, GABA$_B$R1/GABA$_B$R2, GABA$_B$R1/γ2S, or GABA$_B$R1/α1β2γ2S. The responsiveness of each group of oocytes to various concentrations of baclofen was then monitored by using 2-electrode voltage clamping. As in the ERK assay, GABA$_B$R1 alone, GABA$_B$R1/γ2S, and GABA$_B$R1/α1β2γ2S were incapable of activating GIRK currents even at high agonist concentrations. In contrast, oocytes injected with GABA$_B$R1/GABA$_B$R2 exhibited robust GIRK-mediated currents in response to baclofen (Fig. 7*C*). Thus, two different techniques provided evidence that hetero-oligomerization with GABA$_A$ receptors does not confer G protein-dependent signaling to GABA$_B$R1 in the absence of GABA$_B$R2. These observations are consistent with previous reports that have shown GABA$_B$R2 to be the G protein binding partner of the GABA$_B$ receptor heterodimer (13–15). We extended the oocyte studies to examine the possibility that the γ2S subunit might affect the potency of GABA at GABA$_B$ receptors. However, no significant differences were observed in the potency of GABA for GABA$_B$ receptors (GABA$_B$R1/GABA$_B$R2) expressed in the absence (EC_{50} = 4.5 ± 0.6 μM, n = 5) or presence of the γ2S subunit (EC_{50} = 4.5 ± 0.6 μM, n = 7) (Fig. 7*D*).

The γ2S Subunit Confers Agonist-dependent Internalization to GABA$_B$ Receptors—It has been reported that GABA$_B$ receptors do not undergo agonist-dependent endocytosis or desensitization in HEK-293 cells, although they do desensitize to a significant degree in response to agonist stimulation in cerebellar granule neurons (42). GABA$_A$ receptors, on the other hand, are known to undergo constitutive clathrin-mediated endocytosis in both native and transfected cells via association with adaptor protein complex 2 (AP2) adaptins (43, 44). Interestingly, the γ2S subunit has been shown to constantly recycle between the cell surface and the cytoplasm when expressed alone and to also play a key role in GABA$_A$ receptor internalization (38, 43). Since we found that the γ2S subunit associates with GABA$_B$ receptors, we examined whether it might alter the ability of these receptors to undergo endocytosis. Agonist-promoted internalization of GABA$_B$ receptors was studied in HEK-293 cells in the absence and presence of co-expression with the γ2S subunit using the luminometer-based cell surface expres-

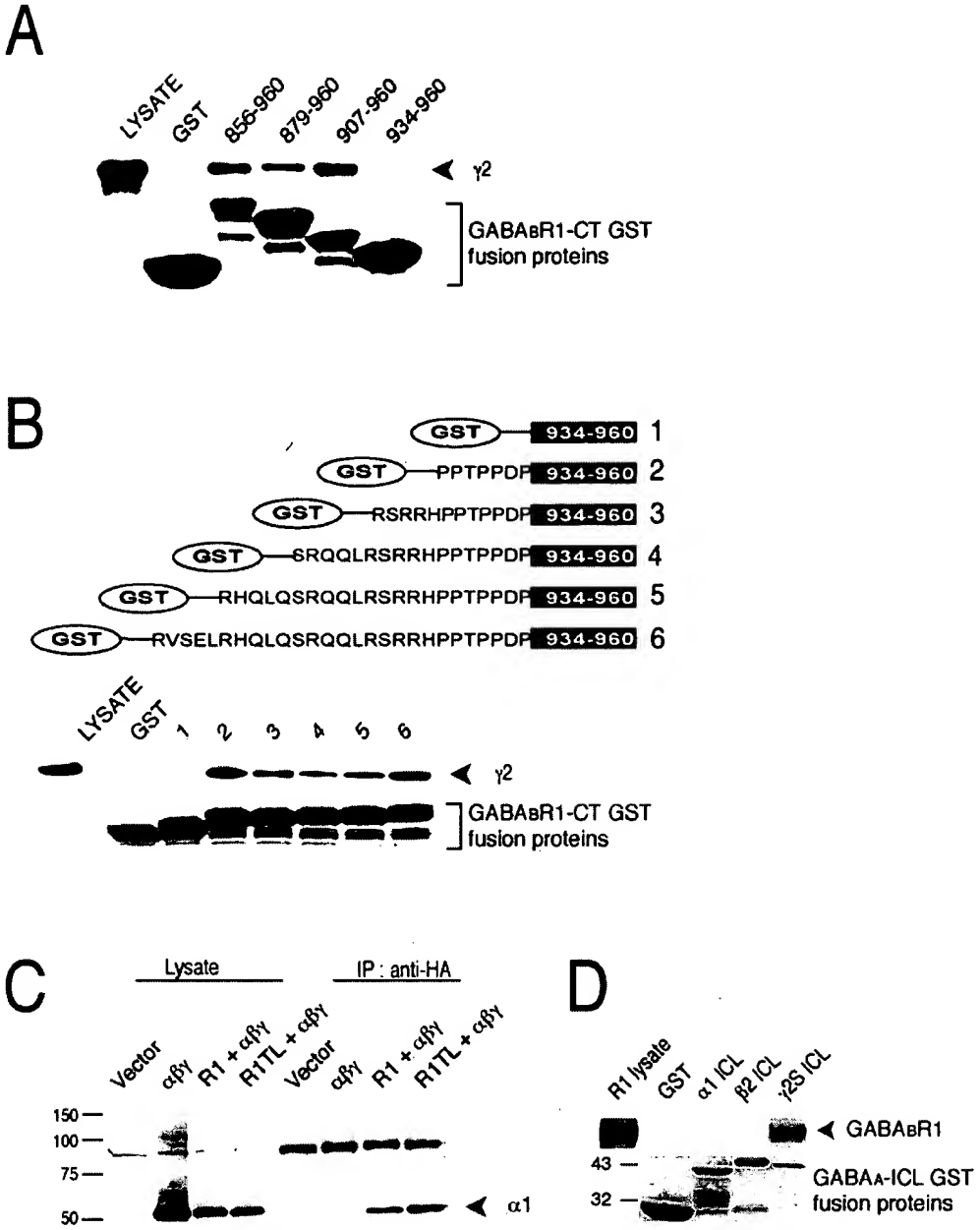

FIG. 5. **The GABA$_B$R1 C terminus is sufficient but not necessary for association with the GABA$_A$ receptor.** *A*, discrete region of the GABA$_B$R1-CT mediates interaction with GABA$_A$ receptors. GST fusion proteins of GABA$_B$R1-CT (amino acids 856–960) and three truncations made from the N-terminal end, corresponding to amino acids 879–960, 907–960, and 934–960 of the full-length receptor, were incubated with solubilized rat brain lysates and assessed for their ability to pull down GABA$_A$ receptor immunoreactivity. Western blot analyses using an antibody against the γ2 subunit indicated that GABA$_A$ receptors were not pulled down by control GST but were markedly pulled down by the full-length GABA$_B$R1-CT. Truncations 879–960 and 907–960 also pulled down GABA$_A$ receptor immunoreactivity, whereas truncation 934–960 did not. These data indicate that a key region of interaction lies between amino acids 907–934 of GABA$_B$R1. The relative sizes and loading levels of the various GABA$_B$R1-CT-GST truncations are shown in the Coomassie-stained gel pictured in the *lower portion* of this panel. *B*, the motif PPTPPDP is important for the interaction of GABA$_B$R1-CT with GABA$_A$ receptors. Additional GABA$_B$R1-CT fusion proteins were created by adding amino acids between 907 and 934 to the 934–960 truncation in increments of 5–7 residues (1–6). GST pull-down experiments with these truncations revealed that addition of the motif PPTPPDP to the 934–960 truncation restored the association with GABA$_A$ receptors. *C*, the GABA$_B$R1-CT is not necessary for GABA$_B$R1 association with GABA$_A$ receptors. Full-length GABA$_B$R1 (*R1*) and GABA$_B$R1 lacking the C terminus (*R1TL*) were both able to immunoprecipitate (*IP*) GABA$_A$ receptor subunit immunoreactivity from HEK-293 cells transfected with either full-length HA-GABA$_B$R1 or the HA-GABA$_B$R1TL mutant along with α1β2γ2S GABA$_A$ receptors. *D*, the ICL of the γ2S subunit is sufficient to mediate the interaction with GABA$_B$R1. Control GST as well as the ICL regions of α1, β2, and γ2S subunits fused to GST were incubated with transfected HEK-293 cell lysates and assessed for their ability to pull down GABA$_B$R1. The γ2S ICL pulled down significant GABA$_B$R1 immunoreactivity, whereas control GST alone, α1 ICL, and β2 ICL did not detectably pull down GABA$_B$R1. All data from the panels of this figure are representative of at least three independent experiments.

sion assay. When GABA$_B$R1 and GABA$_B$R2 were expressed alone, no change in receptor surface expression was observed following a 30-min stimulation with 100 μM GABA, consistent with previous reports of a lack of agonist-induced GABA$_B$ receptor internalization in HEK-293 cells (42). Strikingly, however, GABA$_B$R1/GABA$_B$R2 receptors exhibited robust internalization (>10%) when co-expressed with the γ2S subunit and stimulated with GABA under identical conditions (Fig. 7*E*). These data reveal that co-expression with the GABA$_A$ receptor γ2S subunit confers the capacity for agonist-

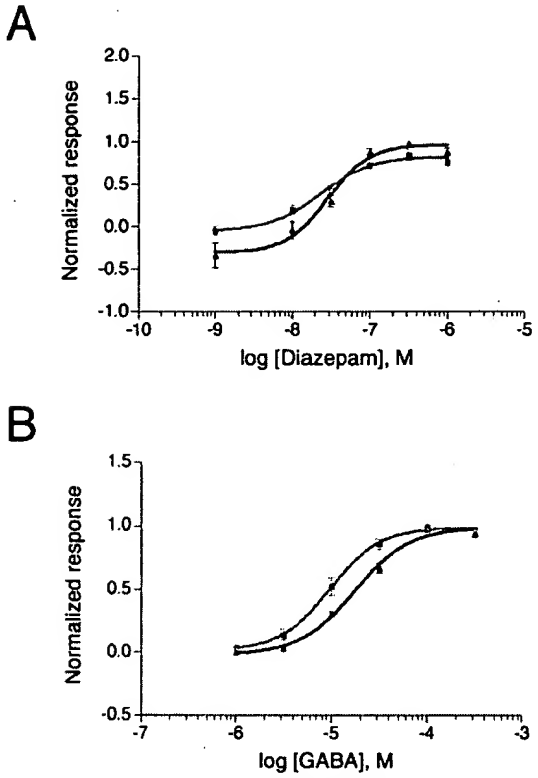

FIG. 6. **GABA$_B$R1 co-expression with GABA$_A$ receptors alters the potency of GABA but not diazepam.** *A*, oocytes were injected with cRNAs encoding GABA$_A$ receptor subunits ($\alpha1\beta2\gamma2S$), in the absence (*triangles*) or presence (*squares*) of co-injected GABA$_B$R1 cRNA. The oocytes were stimulated with a single concentration of GABA (10 μM) and increasing concentrations of diazepam (1, 10, 30, 100, 300, and 1000 nM). The EC_{50} for diazepam modulation of GABA$_A$ receptor-mediated current in oocytes expressing GABA$_A$ receptors alone was not significantly different from EC_{50} values derived from oocytes in which GABA$_A$ receptors were co-expressed with GABA$_B$R1. *B*, oocytes were stimulated with increasing concentrations of GABA (1, 3, 10, 30, 100, and 300 μM). The EC_{50} for GABA-induced currents in oocytes expressing GABA$_A$ receptors alone was significantly lower ($p < 0.01$) than EC_{50} values for GABA-induced currents in oocytes co-expressing GABA$_A$ receptors with GABA$_B$R1. Data are represented as mean ± S.E., as determined in 5–10 independent experiments.

promoted internalization to GABA$_B$ receptors expressed in heterologous cells.

DISCUSSION

It has been shown previously that the GPCR GABA$_B$R1 requires co-expression with another GPCR, GABA$_B$R2, for efficient trafficking to the plasma membrane (7, 9–11). Here we show that a ligand-gated ion channel subunit, the GABA$_A$ receptor $\gamma2S$ subunit, can promote the cell surface expression of GABA$_B$R1 in the absence of GABA$_B$R2. Although we did not find any evidence that GABA$_B$R1 and the $\gamma2S$ subunit can form functional receptors by themselves, we did observe that co-expression of GABA$_B$R1/GABA$_B$R2 with the $\gamma2S$ subunit allows for agonist-promoted internalization of the GABA$_B$ receptors. These findings reveal that the GABA$_A$ receptor $\gamma2S$ subunit regulates GABA$_B$ receptor trafficking in multiple ways, both promoting GABA$_B$R1 surface expression in the absence of GABA$_B$R2 as well as enhancing GABA$_B$R1 endocytosis in the presence of GABA$_B$R2.

Because GABA$_A$ and GABA$_B$ receptors are both activated by GABA, our observations that these two receptor types can associate in cells suggests a mechanism whereby neuronal responses to GABA may be coordinated through the formation of complexes containing multiple GABA receptor subtypes. There have been a handful of previous reports (33–35) describing heterodimerization between GPCRs and ligand-gated ion channels as a mechanism of receptor-receptor cross-talk, but these earlier studies described interactions between receptors activated by distinct neurotransmitters. GABA$_A$ and GABA$_B$ receptors are not only activated by the same transmitter, they are also both found concentrated at symmetric post-synaptic junctions and extensively co-localized in many regions of the mammalian central nervous system (45, 46). In addition to being co-localized at symmetric synapses, GABA$_A$ and GABA$_B$ receptors may also be found together extrasynaptically, as it has been shown that a significant fraction of neuronal GABA$_A$ receptor $\gamma2$ subunits (47) and GABA$_B$ receptors (45, 48, 49) are expressed in extrasynaptic regions. Our co-immunoprecipitation experiments revealed a robust association between GABA$_A$ receptor $\gamma2$ subunits and GABA$_B$ receptors derived from native brain tissue, strongly suggesting that this interaction does occur *in vivo*.

There are numerous reports describing examples of cross-talk between GABA$_A$ and GABA$_B$ receptors for which the molecular mechanisms are obscure, notably the ability of GABA$_A$ and GABA$_B$ receptors to mutually influence each other's ligand binding properties (23–25) and signaling activity (24, 26–29). Numerous precedents in the literature illustrate that modulation of signaling, endocytosis, and/or pharmacology can result from direct interactions between receptors (30, 31, 33–35). The physical association between GABA$_A$ and GABA$_B$ receptors that we have observed may play an analogous role in underlying physiological cross-talk between the two receptor types. Along these lines, we found that co-expression with GABA$_B$R1 modestly increased the potency of GABA acting at GABA$_A$ receptors expressed in oocytes. This change in properties might be due to the physical association between the two receptors directly producing a conformational change in the GABA$_A$ receptor complex that enhances its affinity for GABA, or alternatively might be due to an indirect influence of GABA$_B$R1 on interactions between the various GABA$_A$ receptor subunits. It is well known that GABA$_A$ receptor binding sites are extremely heterogeneous in brain tissue due to the large amount of GABA$_A$ receptor subunit diversity (2, 50). Our data indicate that GABA$_A$ receptor association with GPCRs such as the GABA$_B$ receptor can subtly modulate GABA$_A$ receptor properties and thereby serve to further increase the functional heterogeneity of GABA$_A$ receptors in the brain. GABA$_A$ receptor functional diversity may also be enhanced via interactions with cytoplasmic proteins such as GABARAP and gephyrin (51, 52), which are known to regulate various aspects of GABA$_A$ receptor clustering, trafficking, and function. Because GABARAP and gephyrin are known to associate with $\gamma2$ subunits, these proteins may also regulate GABA$_A$ receptor function by influencing receptor interactions with GPCRs such as dopamine receptor 5 (33) and GABA$_B$ receptors.

We found that association with the GABA$_A$ receptor $\gamma2S$ subunit has profound effects on GABA$_B$R1 subcellular trafficking in at least two distinct ways. First, we observed that the $\gamma2S$ subunit promotes GABA$_B$R1 cell surface expression in the absence of GABA$_B$R2. Interestingly, the "PPTPPDP" motif on the GABA$_B$R1-CT that we found to be involved in the GABA$_B$R1/$\gamma2S$ interaction is located in close proximity to the "RSRR" motif that has been identified as a key ER retention signal for GABA$_B$R1 (7, 8). This ER retention signal may perhaps be masked by interaction with the $\gamma2S$ subunit in the same manner in which the C-terminal association of GABA$_B$R2 with GABA$_B$R1 is believed to mask the signal, thereby promoting the plasma membrane expression of GABA$_B$R1 (7, 8). However, like the physical interaction between GABA$_B$R1 and

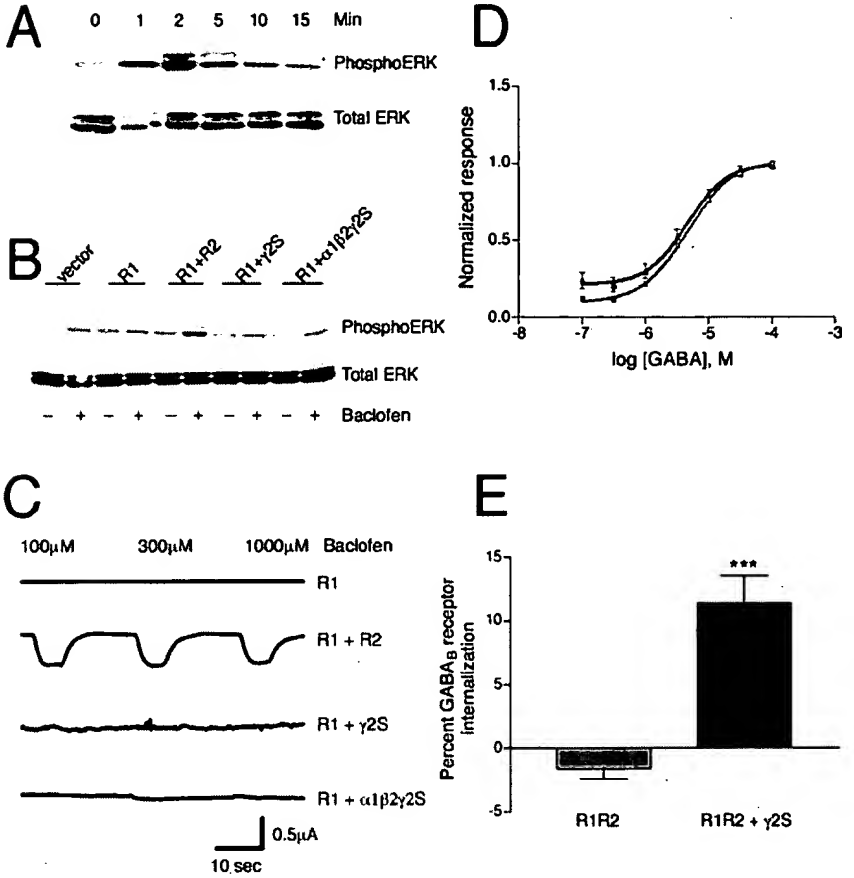

FIG. 7. **Association with the GABA$_A$ receptor γ2S subunit does not confer functionality to GABA$_B$R1 but does confer agonist-promoted internalization to GABA$_B$R1/GABA$_B$R2.** *A*, GABA$_B$ receptors can stimulate ERK phosphorylation in HEK-293 cells. GABA$_B$R1 and GABA$_B$R2 were transfected into HEK-293 cells and stimulated with 100 μM baclofen for 1, 2, 5, 10, or 15 min. A robust enhancement of phospho-ERK immunoreactivity was observed between 2 and 5 min as shown in the *upper panel*. Immunoreactivity for total ERK is shown in the *lower panel*. *B*, neither GABA$_B$R1 alone nor GABA$_B$R1 plus the γ2S subunit can activate ERK upon agonist stimulation. HEK-293 cells were transfected with empty vector, GABA$_B$R1 alone, GABA$_B$R1/GABA$_B$R2, GABA$_B$R1/γ2S, or GABA$_B$R1/α1β2γ2S. The various batches of transfected cells were either left untreated or stimulated with baclofen for 5 min. As shown in *A*, the brief stimulation with baclofen induced a significant increase in ERK phosphorylation for cells transfected with GABA$_B$R1 and GABA$_B$R2 but did not significantly alter ERK phosphorylation levels for all other cells. These data are representative of three independent experiments. *C*, GABA$_B$R1 plus the γ2S subunit cannot activate GIRK currents upon agonist stimulation. Oocytes were injected with GABA$_B$R1 alone, GABA$_B$R1/GABA$_B$R2, GABA$_B$R1/γ2S, or GABA$_B$R1/α1β2γ2S. GIRK1 and GIRK4 potassium channels were co-injected for all conditions. The GIRK currents were recorded following application of 100 μM, 300 μM, and 1 mM baclofen. Large GIRK-mediated currents were observed in oocytes injected with GABA$_B$R1/GABA$_B$R2 but were not observed in oocytes injected with GABA$_B$R1 alone, GABA$_B$R1/γ2S, or GABA$_B$R1/α1β2γ2S. *D*, co-expression with the γ2S subunit does not alter the potency of GABA at GABA$_B$R1/GABA$_B$R2 receptors. Concentration-response curves for GABA were constructed from oocytes injected with either GABA$_B$R1/GABA$_B$R2 (*squares*) or GABA$_B$R1/GABA$_B$R2/γ2S (*triangles*). No significant differences were observed in EC_{50} values for GABA activation of GABA$_B$R1/GABA$_B$R2 *versus* GABA$_B$R1/GABA$_B$R2/γ2S. Data are represented as mean ± S.E. *E*, co-expression with the γ2S subunit confers agonist-promoted internalization to GABA$_B$ receptors. HEK-293 cells were transfected with GABA$_B$R1/GABA$_B$R2 in the presence and absence of the GABA$_A$ receptor γ2S subunit. The cells were stimulated with 100 μM GABA for 30 min at room temperature, and the internalization of GABA$_B$ receptors was monitored by tracking the HA-tagged GABA$_B$R1 subunit with an anti-HA antibody. When examined in the absence of the γ2S subunit co-expression, the GABA$_B$ receptors did not detectably internalize in response to the GABA treatment. In contrast, the GABA$_B$ receptors co-expressed with the γ2S subunit exhibited robust internalization in response to the same agonist stimulation. Data are represented as mean ± S.E. (*** = $p < 0.001$). All data from the panels of this figure are representative of 3–10 independent experiments.

GABA$_B$R2, the association between GABA$_B$R1 and the γ2S subunit is not restricted to the intracellular regions of the two proteins and probably involves the transmembrane domains as well, as indicated by our co-immunoprecipitation experiments where the GABA$_B$R1 mutant lacking the C terminus was found to associate with GABA$_A$ receptors almost as well as wild-type GABA$_B$R1. A second effect of the GABA$_B$R1/γ2S interaction is that co-expression with the γ2S subunit can confer to GABA$_B$R1/GABA$_B$R2 receptors the ability to internalize in response to agonist stimulation. Agonist-promoted internalization of GPCRs is known to play a key role in the regulation of receptor desensitization and resensitization (53), but GABA$_B$ receptors expressed in heterologous cells have been found to neither internalize nor desensitize in response to agonist treatment (42). Because GABA$_B$ receptors are known to exhibit robust desensitization in cerebellar granule cells (42), it seems likely that neurons must express one or more regulatory proteins that are absent from HEK-293 cells and required for agonist-promoted internalization and desensitization of GABA$_B$ receptors. Examples of such regulatory proteins may include G protein-coupled receptor kinase-4 (42) and the GABA$_A$ receptor γ2S subunit, which is abundantly expressed in cerebellar granule cells (54) and, according to our data, capable of conferring agonist-promoted internalization to heterologously expressed GABA$_B$ receptors.

In summary, our findings reveal that the γ2S subunit plays a dual role in the trafficking of GABA$_B$ receptors. The γ2S subunit both supports GABA$_B$R1 cell surface expression in the absence of GABA$_B$R2 and also facilitates the removal of GABA$_B$ receptors from the cell surface upon agonist stimulation. Both

of these actions are consistent with observations that the γ2S subunit exhibits constitutive recycling between the plasma membrane and intracellular compartments when expressed alone in heterologous cells (38, 43). In addition to regulating $GABA_B$ receptor trafficking, the interaction between $GABA_A$ and $GABA_B$ receptors reported here also provides a novel mechanism potentially underlying cross-talk and mutual regulation between these two different classes of GABA receptor.

Acknowledgments—We thank Amanda Castleberry, Anthony Lau, and Asha Shah for technical assistance; Antoine Almonte for oocyte preparation; Steve Traynelis and the members of his lab for providing oocyte recording chambers and advice; David Mott for helpful discussions; and all of the investigators who supplied receptor cDNAs to facilitate this work (as listed under the "Materials and Methods").

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